

Please replace the paragraph at page 10, line 28, with the following paragraph:

a² Two consensus polyadenylation signals were also found: ATTAA (SEQ ID No. 10) (bp 1805)) and AATAA (SEQ ID No. 11); (bp 2083)), which were situated 15 nucleotides upstream from the poly(A) tail (Breathnach et al., Ann. Rev. Biochem. 50:349-383 (1981)).

Please replace the paragraph beginning at page 10, line 33, with the following paragraph:

a³ The amino acid sequence of adult human liver FMO (form 3) deduced from the cDNA clone is shown in SEQ ID No. 8. Comparison of HLFMO 3 with human liver FMO form 1 (HLFMO 1) (Dolphin et al., J. Biol. Chem. 266:12379-12385 (1991)), pig liver FMO form 1 (PLFMO) (Gasser et al., Biochemistry 29:119-124 (1990)), rabbit liver FMO form 1 (RLFMO I) (Lawton et al., J. Biol. Chem. 265:5855-5861 (1990)) and rabbit lung FMO (RLuFMO) (Lawton et al., J. Biol. Chem. 265:5855-5861 (1990)) showed only a modest degree of primary sequence identity (e.g., 53-57%). HLFMO 3 contained a putative FAD binding domain at amino acid residues 9-14 (e.g., GAGVSG (SEQ ID No. 13)) and a putative NADP⁺ binding domain at residues 191-196 (e.g., GLGNSG SEQ ID No. 14)). These cofactor-binding regions were highly conserved among all of the mammalian FMO enzymes known as well as the FMO bacterial equivalent, cyclohexanone monooxygenase (Chen et al., J. Bacteriol. 179:781-789 (1988)). In contrast to other mammalian hepatic FMO forms, HLFMO 3 has only a single putative consensus N-glycosylation site (Asn-Xxx-Ser/Thr) at residues 61-63. It was notable that HLFMO 3 did not contain the putative N-glycosylation sites at residues 120-123 and 315-317 that were present in form 1 FMOs.

Please replace the paragraph beginning at page 17, lines 33, with the following paragraph:

a⁴ The invention provides isolated and purified polynucleotide molecules encoding FMO3 capable of hybridizing under stringent conditions to an oligonucleotide of 15 or more contiguous nucleotides of SEQ ID NO: 7 or SEQ ID NO: 9 and their complementary strands. The

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isolated FMO3 polynucleotide molecules preferably encode FMO3 proteins or fragments thereof that have enzymatic activity.

Please replace the paragraph beginning at page 19, line 25, with the following paragraph:

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As would be evident to one of ordinary skill in the art, the polynucleotide molecules of the present invention may be expressed in *Saccharomyces cerevisiae*, filamentous fungi, and *E. coli*. Methods for expressing cloned genes in *Saccharomyces cerevisiae* are generally known in the art (see, "Gene Expression Technology," Methods in Enzymology, Vol. 185, Goeddel (ed.), Academic Press, San Diego, CA, 1990 and "Guide to Yeast Genetics and Molecular Biology," Methods in Enzymology, Guthrie and Fink (eds.), Academic Press, San Diego, CA, 1991; which are incorporated herein by reference). Filamentous fungi (e.g., strains of Aspergillus) may also be used to express the proteins of the present invention. Methods for expressing genes and cDNAs in cultured mammalian cells and in *E. coli* is discussed in detail in Sambrook et al. (Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; which is incorporated herein by reference). As would be evident to one skilled in the art, one could express the protein of the instant invention in other host cells such as avian, insect and plant cells using regulatory sequences, vectors and methods well established in the literature.

Please replace the paragraph beginning at page 24, line 14, with the following paragraph:

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As shown in Table 1, N-oxygenation of chlorpromazine and compounds 2-4 was detectable, but compounds 5 and 6, with longer side chains, were better substrates for human liver microsomes and solubilized protein from *E. coli* transformed with pTrcHLFMO 3. Dichloromethane extracts of metabolic incubations with microsomes and solubilized protein of selected substrates from transformed *E. coli* were subjected to mass spectral analyses. The liquid secondary ion mass spectra (+LSIMS) of the tertiary amine N-oxide metabolite of compounds isolated from pig liver microsomes was similar with the chemical ionization (CI) spectrum of the N-oxide metabolite isolated from human liver microsomes and solubilized protein from *E. coli* transformed with pTrcHLFMO 3 (Table 2A, B).

Please replace the paragraph beginning at page 39, line 25, with the following paragraph:

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Mercapturates and S-cysteine conjugates of chemicals, drugs, and biologicals are substrates for FMO from animals and humans. The S-oxide products are not indefinitely stable and, depending on the functional groups present, eliminate or rearrange to other products. The by-products are selective biomarkers for the S-oxygenation of chemicals by FMO. For example, a S-cysteine conjugate of an isoprene moiety (farnesylcysteine methyl ester) is an excellent substrate for FMO. The S-oxide is not stable and the farnesyl group is cleaved. Because farnesylation of ras proteins is required for oncogene products, FMO-mediated S-oxidation of farnesylated proteins may be protective against cancer. Further, by-products of the S-oxidation of farnesylated ras proteins could be useful bioindicators of protection against cancer.

Please replace the paragraph beginning at page 41, line 36, with the following paragraph:

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After administration to humans, 2% of a dose of verapamil is recovered unchanged. However, only 50% of a dose is accounted for in terms of identifiable metabolites. Verapamil is metabolized to the N-oxide which undergoes Cope-type elimination to produce novel metabolites. Screening with cDNA-expressed HLFMO obviates whether this is the case and whether the metabolite produced (i.e., styrene) is responsible for some of the toxicities associated with administration of verapamil. This is another example of the usefulness of HLFMO 3 in drug design.

Please replace the paragraph beginning at page 45, line 30, with the following paragraph:

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The adult human liver λ -gt11 cDNA library was obtained from Clontech (Mt. View, CA) and the adult human liver λ -gt11 library was constructed by standard procedures (Huynh et al., DNA Cloning: A Practical Approach (IRL, Oxford, U.K.), 49-78 (1985)). The cDNA libraries were screened by an in situ hybridization technique (Benton & Davis, Science 316:180-182 (1987)) at a density of 100,000 pfu/132-mm-diameter plate. Approximately 2×10^6

plaques were examined. The probe used was a mixture of three oligonucleotides complementary to the pig liver FM0 (PLFM0) cDNA (Gasser et al., Biochemistry 29: 119-124 (1990)). The three 36-mer oligonucleotide probes were prepared by the UCSF Biomolecular Resource Center with an Applied Biosystems Model 380B DNA synthesizer using phosphoramidite chemistry. PLFM01, a 36-mer of sequence 5'-ATCGCTCCTCTCAAAGCAGGTGGGCTCCAGCCTTC-3' (SEQ ID No. 14), is complementary to the pig liver cDNA nucleotide sequence 127-162, PLFM02 a 36-mer of sequence 5'-CTCATCAAGGGGAAAGCAAAGGTGTATCCAGT-3' (SEQ ID No. 1), is complementary to the pig liver cDNA nucleotide sequence 1041-1076 and PLFM03, a 36-mer of sequence

5'-GAATGTTCGGTCCCACTGGGTCATGATGATAGCATTCCT-3' (SEQ ID No. 2), is complementary to the pig liver cDNA nucleotide sequence 1509-1545. Purified oligonucleotides were 5'-end-labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. Filters from the first screening were prewashed in 50 mM Tris HCl (pH 8.0), 1M NaCl, 1mM EDTA, and 0.1% SDS for 2 h at 42°C. Pre-hybridization was carried out in 6 x SSC, 0.5% N-lauroyl sarcosine, 10 x Denhardt's solution and 50 mg/ml of denatured salmon sperm DNA for 4 h at 45°C. The 32 P-labeled probes (10^6 cpm/filter) were added to the same prehybridization buffer without N-lauroyl sarcosine and hybridization was carried out for 16 h at 45°C. The filters were washed five times in 2 x SSC containing 0.1% SDS for 10 min at room temperature, twice for 1 h at 55°C, dried and exposed to Kodak X-Omat AR5 films at -80°C for 24 h with intensifying screens. The positive clones were further purified by additional screenings under the same hybridization conditions.

Please replace the paragraph beginning at page 48, line 20, with the following paragraph:

To obtain the full-length open reading frame for HLFMO 3 containing convenient ends for subcloning into the expression vector pTrc99A, the PCR technique was employed. For the PCR reaction, two oligonucleotide primers were designed. Primer A (5'-GGTACCACATGTCCATGGGGAAGAAAG-3' (SEQ ID No. 3) consisted of an Afl III site at the 5'-end of the HLFMO 3 cDNA. Primer B (5'-GACGTCGACGGATCCTTAGGTCAACACA-3' (SEQ ID No. 4) had a Sal I site at the 5'-end and a 13 nucleotide sequence complementary to the 3'-end of the HLFMO-3 cDNA coding strand. The primers were synthesized with a Biosearch

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8600 DNA synthesizer (Applied Biosystems, Foster City, CA) using phosphoramidite chemistry and purified on oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA). The PCR was carried out with a Perkin-Elmer Cetus (San Jose, CA) DNA thermal cycler, using a GeneAmp DNA amplification reagent kit with the largest HLFMO3 cDNA clone from λ -gt11 as a template. Thirty thermal cycles were performed, and each cycle included a denaturation step at 98°C for 1 sec, an annealing step at 50°C for 15 sec, and an extension step at 60°C for 4 min. After 30 cycles of PCR reaction, a full-length HLFMO 3 cDNA coding strand was obtained with Afl III restriction site and a Sal I site attached to the 5'-and 3'-ends of the cDNA, respectively. The entire PCR insert was sequenced. The 5' and 3' -ends were also sequenced.

Please replace the paragraph beginning at page 49, line 15, with the following paragraph:

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After the PCR product was purified and digested with Afl III and Sal I restriction enzymes, the generated fragment was subjected to fractionation by electrophoresis on 1% agarose gel, purified and subcloned into the Nco I-Sal I sites of the inducible prokaryotic expression vector pTrc99A. The pTrc99A vector contained the lacZ ribosome-binding site and was driven by the strong hybrid trp/lac promoter (i.e., pTrc) under the control of the lacI^q allele of the lac repressor gene. The recombinant clones were identified by screening with a ³²P labeled human FMO3 cDNA probe (Rigby et al., J. Mol. Biol. 113:237-251 (1977)) using the Afl III-Sal I generated PCR fragment, and the correct insertion was confirmed by digestion with Nco I and Sal I and DNA sequence analysis.

Please replace the paragraph beginning at page 51, line 35, with the following paragraph:

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The cis- and trans-diastereomeric S-oxides of (+) and (-)-4-bromophenyl-1,3-oxathiolane were separated and quantified by HPLC as previously described (Cashman et al., J. Amer. Chem. Soc. 112, 3191-3195 (1990)). HPLC of the S-oxide diastereomers with a Chiracel OT chiral analytical column (25 cm x 0.15 cm inner diameter, Daicel Chemical Co., New York, NY) was accomplished with a Beckman system, Kratos UV detector (240 nm) and a Hewlett Packard Integrator. The mobile phase consisted of methanol/isopropyl alcohol/hexane (1:10:89,

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v/v) which efficiently separated (+)-4-bromophenyl-1,3-oxathiolane, (+)-trans-4-bromophenyl-1,3-oxathiolane S-oxide, and (+)-cis-4-bromophenyl-1,3-oxathiolane S-oxide which had retention volumes of 5.4, 10.5, and 18.8 ml, respectively. The system also efficiently separated (-)-4-bromophenyl-1,3-oxathiolane, (-)-trans-4-bromophenyl-1,3-oxathiolane S-oxide, and (-)-cis-4-bromophenyl-1,3-oxathiolane S-oxide which had retention volumes of 5.4, 9.6 and 11.8 ml, respectively. Quantification was accomplished by comparing integrated HPLC peak areas after taking into account the difference in the extinction coefficients at 240 nm. Likewise, the cis and trans S-oxide diastereomers of (+)- and (-)-2-methyl-1,3-benzodithiole were separated with a Chiracel OT chiral analytical column. The mobile phase consisted of methanol/isopropyl alcohol/hexane (0.25:2.8:7.25, v/v) which efficiently separated 2-methyl-1,3-benzodithiole and the (+)-(1R,2S), (-)-(1S,2R), (+)-(1R,2R), and (-)-1S,2S) S-oxide diastereomers which had retention volumes of 17.6, 19.4, 23.6, and 24.5 ml, respectively.

Please replace the paragraph beginning at page 57, line 34, with the following paragraph:

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For subcloning HLFMO3 into the maltose binding protein fusion system, two oligonucleotides were synthesized to use in PCR amplification of the HFMO3 cDNA. This allowed proper insertion of HFMO3 cDNA into the expression vector pMAL-c2 in such a way that allowed for the fusion of HFMO3 cDNA at the 3' end of sequences encoding the maltose binding protein (MBP). Oligos 5'-GGGAAGAAAGTGGCCATC-3' (SEQ ID No. 15) and 5'-CCGGTCGACGGATCCAAGCTTAGGTCAACACAAGG-3' (SEQ ID No. 16) were the 5' and 3' PCR primers, respectively. The 3' oligo includes HindIII, BamHI and SalI sites which allowed, among other manipulations, insertion of the PCR fragment into the pMAL-c2 vector between the XmnI site (blunt end) and the HindIII site. The two oligonucleotides were used in an amplification reaction with the HFMO3 cDNA-containing vector pHFMO3-f1. Approximately 0.1 µg of the single-stranded form of pHFMO3-f1 was used as the template for PCR. 100 pmol of each primer was used in a 50 µl reaction under standard PCR buffer conditions. Cycle conditions were 94°C melting, 55°C annealing and 74°C extension and after 20 cycles, yielded sufficient DNA to proceed with the cloning steps. The PCR product was treated with DNA polymerase I (Klenow fragment) to ensure that the DNA fragments had flush ends prior to cloning. The vector, named

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pMAL-HFMO3, is the result of cloning the PCR fragment into the XmnI and Hind III sites of the vector pMAL-c2. Several clones were examined for expression before one was isolated that produced a product with the expected size of the full length fusion. The junction between the maltose binding protein and HFMO3 contains a factor Xa cleavage site and is a feature of the pMAL-c2 vector. The fusion junction was designed so that cleavage of the HFMO3-MBP fusion protein with factor Xa would release an HFMO3 product that contains an amino-terminal glycine, which is the native condition of HFMO3 isolated from human liver.

Please replace the paragraph beginning at page 58, line 30, with the following paragraph:

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The lysine 158 codon of HFMO3 is AAG. A single change to GAG converts this position to a codon for glutamate. This was accomplished by site-directed mutagenesis using the BioRad Mutagene kit. The oligonucleotide synthesized for this purpose was: 5'-CC TGG AAA GGA CT(G/C) TTT TGG TAG GTT GGG-3' (SEQ ID Nos. 17 and 18, respectively). This oligo was designed to change lysine 158 to either a glutamate or glutamine. In fact only glutamate was recovered in several attempts verified by sequencing of candidates. The mutagenesis was carried out on a subclone of HFMO3 (NcoI to SacI) in vector pGEM(-). The recovered mutation was then transferred to the MBP-fusion expression vector by exchanging the NheI to SacI fragment of the mutagenized vector with that of vector pMAL-HFMO3.

Please replace the paragraph beginning at page 61, line 18, with the following paragraph:

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After purification of the HFMO3-MBP by amylose resin affinity chromatography, the amount of detergent present was analyzed by extraction of the protein and HPLC analysis. This was done for the glu¹⁵⁸-HFMO3-MBP. 185 µg of glu¹⁵⁸-HFMO3-MBP was placed in a typical incubation format and extracted with 1 ml acetonitrile. The organic fraction was mixed thoroughly, separated by centrifugation, evaporated to dryness and taken up in methanol for analysis by HPLC. HPLC was done with a Hitachi HPLC as described above and a C-18 Microsorb reverse phase column (Rainin, Emeryville, CA). An eluent of CH₃CN/H₂O/

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CH₃OH/NH₄OH (62:35:3:0.4; vol:vol) was used to separate Triton X-100 and other detergents (retention volume 10 ml) from other polar materials. Using a standard curve of 254 to 5088 pmol of Triton X-100 it was established that 0.89 nmol of detergent per nmol of HFMO3 was present after the amylose resin affinity chromatography step. This preparation was designated the "detergent free" HFMO3-MBP.

Please replace the paragraph beginning at page 63, line 2, with the following paragraph:

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Expression from the pMAL-c2 derived HFMO3 fusion vectors resulted in considerable overproduction of full length HFMO3-MBP and several other different sized proteins that were retained on the amylose affinity resin. The sizes of these products range from approximately 100 kD (the expected size of the full length fusion product) to approximately 43 kD. These products were present only in cells that contain the fusion vector and were not seen in cells without a vector or with the pMAL-c2 vector (which produces maltose binding protein exclusively as the only amylose binding protein). The common property of these proteins is that they bind to the amylose resin and are specifically released by maltose. It was assumed that these proteins are all the result of expression from the HFMO3-MBP vector, contain the maltose binding protein domain and arise either through proteolytic breakdown of the full length protein or, more likely, are the result of incomplete translation because the SDS-PAGE pattern observed and the relative intensity of the bands was almost always identical from prep to prep, implying an inherent stability of the products in cells or cell extracts. Purification of the maltose binding domain fusion proteins was possible in one step using amylose resin. There was a dramatic increase in specific activity for the conversion of 5-DTP to 5-DTP N-oxide after this step. Further fractionation of the MBP-containing species on the basis of size indicated that the bulk of the HLFMO3 oxygenation activity was associated with the full length product, although the 60 kD protein associated strongly with the full length protein. Based on the performance of the Sephacryl S-300, the active fraction elutes earlier, and is apparently much larger than expected for a 100 kD protein. This suggests that the active form of the enzyme is in a higher ordered complex. In addition to aiding in the purification of HFMO3 enzyme, the addition of the maltose binding protein domain to the amino

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cont terminus of HFMO3 clearly stabilized the enzyme when compared to enzyme purified from liver microsomes or produced as a non-fusion protein in *E. coli*.

Please replace the paragraph beginning at page 66, line 29, with the following paragraph:

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The cDNA-expressed human FMO3-maltose binding protein (FMO3-MBP) efficiently catalyzes the sequential N-oxygenation of primary amines to oximes through the intermediacy of hydroxylamines. Thus, 10-(N-n-octylamino)-2- (trifluoromethyl)phenothiazine is stereoselectively converted to the cis oxime via the intermediate hydroxylamine as determined by the analysis of metabolic extracts by HPLC. A similar product stereoselectivity was observed in the presence of human liver microsomes. Studies on the biochemical mechanism of oxime formation suggests that cis-oxime formation is largely dependent on human FMO3 activity. In addition, formation was not dependent on autoxidation of hydroxylamines or generation of oxygen radical species. Thus, H₂O₂, OH⁻ and O₂⁻ had virtually no effect on the formation of oxime from the primary hydroxylamine. This result is in contrast to what has been reported for other forms of FMO (i.e., FMO1) (Rauckman et al., Mol Pharmacol. 15, 131-137 (1979)) and suggests that FMO3 possesses an intrinsic ability to avoid oxygen radical-mediated substrate oxygenations. This represents a fundamental difference between FMO3 and other FMOs and indicates a use of FMO3 as a stereoselective catalyst in the formation of precious chemicals or hard to form metabolites having great stereochemical purity.

Please replace the paragraph beginning at page 67, line 15, with the following paragraph:

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Similarly, the biogenic amine phenethylamine is stereoselectively converted to trans oximes in the presence of adult human liver microsomes and cDNA-expressed human FMO3-MBP via the intermediacy of the hydroxylamine as determined by analysis of metabolic extracts by HPLC. Studies of the effect of metabolic inhibitors on oxime formation suggested that adult human liver microsomal FMO3 is largely responsible for trans oxime formation. It is notable that in the adult human liver microsomes examined, there is more than a 6-fold increase in the efficiency of forming oximes compared with pig liver microsomes. This is surprising in light of

the fact that pig liver microsomes are generally 10 to 15-fold more active at FMO-mediated formation of tertiary amine N-oxides compared to FMO-mediated formation of tertiary amine N-oxides in adult human liver microsomes. Thus, the prominent FMO enzyme form in pig liver microsomes (i.e., FMO1) is about 60 to 100-fold less active than the major form of FMO in adult human liver microsomes (i.e., FMO3) at forming oximes from primary amines. Depending on the substrate and the source of FMO1, large concentrations of primary amines (i.e., n-octylamine) have been observed to stimulate FMO-like activity. Both variants of human FMO3 (i.e., Lys 158 and Glu 158 variants) were approximately equally efficient at stereoselectively forming trans phenethylamine oxime from phenethylamine (i.e., 3.2 ± 0.4 and 4.9 ± 0.6 nmol trans oxime formed / min / mg of protein, respectively). To confirm the intermediacy of the hydroxylamine metabolite as an obligatory intermediate in the formation of trans oxime, the conversion of phenethyl hydroxylamine to oxime was examined in the presence of human cDNA-expressed FMO3-MBP variants. Similar to phenethylamine, phenethyl hydroxylamine was stereoselectively converted to the trans oxime by human FMO3-MBP variants Lys 158 and Glu 158 (i.e., 3.4 ± 0.3 and 4.5 ± 0.8 nmol trans oxime formed / min / mg of protein, respectively). Formation of oxime product was not a consequence of autooxidative or oxygen radical participation because H_2O_2 , $OH\cdot$ (and $O_2\cdot$) made very little contribution to oxime formation. The use of cDNA-expressed human FMO3 will permit the production of unusual or rare nitrogen-, sulfur-, phosphorous- and other heteroatom-containing chemicals and metabolites possessing a great degree of stereochemical purity. In addition, the observations about biogenic amine metabolism by human FMO3 indicates that FMO3 plays a novel role in amine metabolism and cellular homeostasis. Generation of novel biogenic amine metabolites with pharmacological activity in their own right or truncation or abrogation of biological activity of biogenic amines by the action of FMO3 suggests that FMO3 activity may participate in controlling a number of fundamentally important biochemical pathways. Analysis for the formation or lack of formation of the FMO3-mediated biogenic amine metabolites in humans may serve as a bioindicator of some fundamental abnormality associated with cardiovascular, central nervous system or some other disease state associated with biogenic amine metabolism.

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